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A *CHLORELLA* VIRUS GENE PROMOTER FUNCTIONS AS A STRONG PROMOTER BOTH IN PLANTS AND BACTERIA

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SUMMARY: An upstream region from an algal virus methyltransferase gene was tested for promoter function in transgenic plants, electroporated monocot protoplasts and bacteria. Fusion of the 851 bp upstream region to a reporter gene significantly enhanced the reporter gene expression in transgenic *Arabidopsis* and potato plants and in electroporated maize and *Sorghum* cells relative to the cauliflower mosaic virus 35S promoter. The promoter was also functional in several *Escherichia coli* strains and in three species of phytopathogenic bacteria, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. These findings indicate that the upstream region is a strong promoter uniquely functional in both eukaryotes and prokaryotes and capable of using both eukaryotic RNA polymerase II and bacterial RNA polymerases.

Genetic engineering provides a method for isolating, selectively amplifying, and expressing genes encoding desirable traits. Genes are often obtained from one organism and transferred into another organism to either produce large quantities of a gene product or to improve characteristics or traits of the transformed organism. Expression of a gene introduced into transgenic organisms depends mainly on the promoters used to generate RNA transcripts. With the advent of biotechnology, the necessity of using strong promoters is becoming increasingly important. Furthermore, as most of the gene manipulation is carried out in prokaryotic bacteria, promoters that function both in prokaryotes and eukaryotes have added advantages. There are only a limited number of promoters that are available for expression of foreign genes in plants. Identification of new strong promoters will be invaluable for plant biotechnology. Several strong promoters and hybrid promoters have been identified from bacteria, none of these promoters, however, are functional in plants.

The discovery of viruses that infect eukaryotic *Chlorella*-like green algae has led to the identification of many interesting genes coded for in the genomes of these viruses (1).

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Among these are several type II restriction endonucleases and their cognate methyltransferase genes. During the course of our experiments where attempts were being made to express methyltransferase genes in plants, it was discovered that the promoters of these genes were also functional in plants. In this report we show that the promoter from the viral adenine methyltransferase gene is strongly expressed in transgenic *Arabidopsis* and potato plants and in maize and *Sorghum* suspension culture cells. In addition, the promoter is also expressed in several bacterial species.

MATERIALS AND METHODS

Construction of Plasmids: An 851 bp fragment immediate upstream from the translation start site of the *Chlorella* virus adenine methyltransferase gene (*amt*) (2) was excised, and fused with the chloramphenicol acetyltransferase (*cat*) reporter gene to create the plasmid pAM1029. This plasmid also contained the T-DNA nopaline synthase gene (3) poly-adenylation signal (T_{nos}) following the *cat* gene. A similar construct was made by subcloning a 1956 bp *Hind*III-*Sma*I fragment from plasmid pAM1029 containing the *amt-cat*-T_{nos} fusion into the *Bam*HI site (Klenow treated) of plasmid pLAFR3 (4) creating pAM1030. The same 1956 bp fragment was also inserted into the *Hpa*I site of the binary vector pGA628 (5) to create pAM1031. For the purpose of comparison, identical constructs were made replacing the *amt* promoter in pAM1030 with the *lac* promoter for bacteria and the *amt* promoter in pAM1031 with the cauliflower mosaic virus (CaMV) 35S promoter (6) for plants. These two constructs were designated as pAM1032 and pAM1033, respectively. An additional construct, pAM1034, was made by replacing the *amt* promoter in pAM1029 with the CaMV 35S promoter. Plasmids pAM1029 and 1034 contained ColE1 replicons whereas the rest of the plasmids contained broad host-range replicons.

Transformation of Bacteria: Plasmids pAM1029, pAM1030 and pAM1032 were used for transformation of *E. coli* strains MC1000 (7), JM83 (8), K-802 (9), DH5α (10) and MM294 (11); two species of *Erwinia*, viz., *E. stewartii* (ES), and *E. amylovora* (EA); and *Pseudomonas syringae* pv. *syringae* (PSS) and *Xanthomonas campestris* pv. *phaseoli* (XCP). Bacteria were transformed by two methods, chemical transformation (12) and electroporation (13,14). A Bio-Rad Gene Pulsar apparatus set at 25 μF, 12.5 kV/cm, 200 ohms was used for electroporation.

Plant Transformation: *Arabidopsis thaliana* and potato were stably transformed using plasmids pAM1031 and pAM1033 by the *Agrobacterium* co-cultivation method (15). Transformants were selected on kanamycin to obtain transgenic seedlings. Three to five week old seedlings were assayed for chloramphenicol acetyltransferase (CAT) activity as described in Mitra et al. (16). Protoplasts were prepared from maize (black mexican sweet) and *Sorghum* suspension culture cell lines and electroporated with plasmids pAM1029 and pAM1034 as described in Mitra and An (3). Electroporated cells were incubated for 48 hours and then harvested for CAT assay.

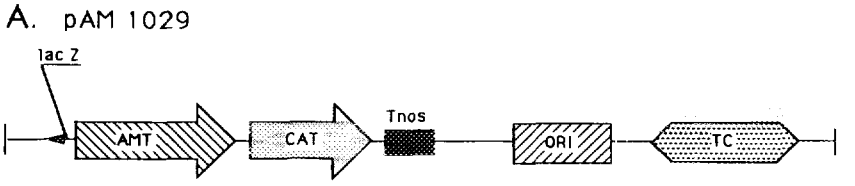
CAT Assay: Individual bacterial colonies were grown in liquid media with appropriate antibiotics. Bacterial cells were harvested by centrifugation and resuspended in 100-200 μl protein extraction buffer (3). Electroporated maize and *Sorghum* protoplasts were also similarly processed. The cells were lysed by sonication and CAT activities were measured with standardized protein extracts (17) as described by Gorman et al. (18). On the basis of preliminary assays, protein concentrations in individual samples were

adjusted in order to obtain CAT activity within its linear range (<40% acetylated fraction). Each strain of bacteria and all four plant species were analyzed for endogenous CAT activity prior to transformation and showed no back-ground CAT activity.

RESULTS AND DISCUSSION

The large, dsDNA-containing, plaque-forming viruses that infect certain, unicellular, eukaryotic, *Chlorella*-like green algae encode several interesting genes, including genes for DNA methyltransferases and DNA restriction endonucleases (1). Since the *Chlorella* viruses are unique among "plant" viruses in having a large (330 kbp) dsDNA genome, certain viral housekeeping genes and regulatory elements might be useful for plant genetic engineering. Consequently, we tested the upstream region of a viral adenine DNA methyltransferase gene for promoter function in plants and bacteria.

Two different constructs, pAM1029 and pAM1030 were made to achieve transcriptional fusion between the *amt* promoter and the *cat* reporter gene. These two plasmids had pUC and pLAFR3 backgrounds, respectively. An additional construct, pAM1032 containing a *lac-cat* fusion, was used to compare *amt* promoter with *lac* promoter. Electroporation of plasmid pAM1029 into five *E. coli* strains resulted in very strong CAT expression in all five strains (Fig. 1). In this plasmid the *amt-cat*-T_{nos} cassette is



B.

HOST	METHOD OF TRANSFORMATION	CAT ACTIVITY
MC1000	EP	2893
JM83	EP	3157
K802	EP	2755
DH5 α	EP	3824
MM294	EP	2092

Figure 1. CAT activity in *E. coli* strains transformed by electroporation (EP) with plasmid pAM 1029 (A). Ten colonies from each strain were individually tested to obtain average CAT activity (B). CAT activities were standardized from known amounts of total protein in various extracts used for CAT assay. One unit of CAT catalyzes acetylation of 1 nM chloramphenicol per min at 37° C. ORI, ColE1 replicon; TC, tetracycline resistance gene. See text for other descriptions.

inverted with respect to the *lac* promoter and no read-through transcription was detected. Plasmid pAM1030, and plasmid pAM1031 (RK2 replicons) were used to transform several *E. coli* strains and phytopathogenic *Erwinia*, *Pseudomonas*, and *Xanthomonas* species. All bacterial species were successfully transformed either by electroporation or chemical transformation and showed positive CAT activity (Figs. 2 and 3). The *amt* promoter demonstrated efficient expression of the *cat* reporter gene in all bacterial species tested. Two phytopathogenic bacteria, *Pseudomonas* and *Xanthomonas* could only be transformed by electroporation using plasmids containing a wide-host range replicon. The expression level was dependent on the type of plasmid and bacterial strain used. The *amt-cat* fusion in ColE1 plasmids showed highest levels of expression, presumably due to the high copy number of this plasmid. Bacterial species transformed with plasmid pAM1030 exhibited lower levels of CAT activity than those transformed with plasmid pAM1029. Transformation of bacteria with similar plasmids, pAM1030 and pAM1032, generated comparable CAT activities by the *amt* and the *lac* promoters. The *Chlorella* viruses have several structural similarities with bacterial phages, they also posses restriction modification systems (1). Therefore, it is conceivable that these viruses perhaps evolved from bacteriophages. This would also explain why the viral *amt* promoter is efficiently expressed in bacteria.

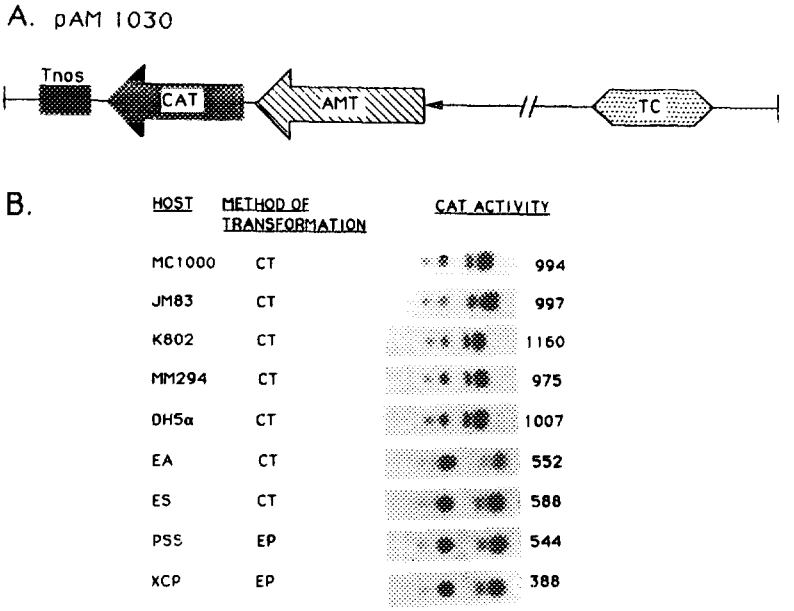


Figure 2. CAT activity in *E. coli* strains and in *Erwinia*, *Pseudomonas* and *Xanthomonas* transformed with plasmid pAM 1030 (A). Ten colonies from each strain were individually tested to obtain average CAT activity (B). CT, chemical transformation. See Figure 1 and text for other descriptions.

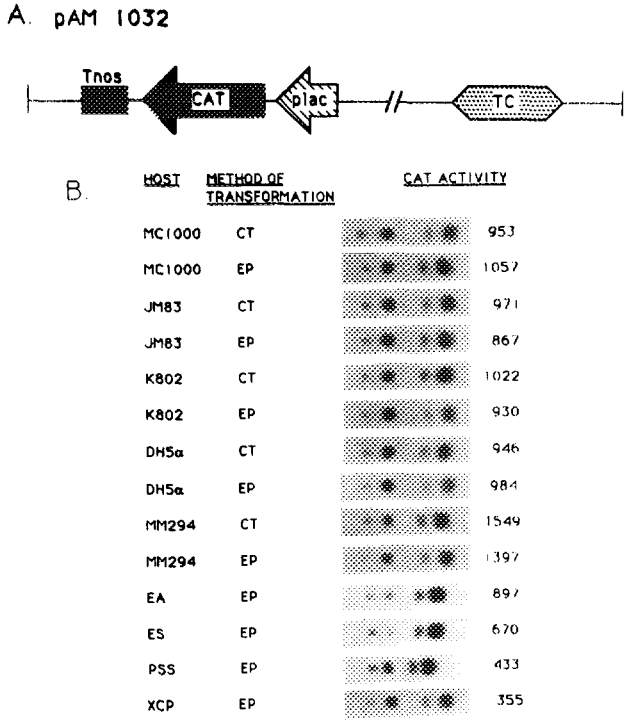
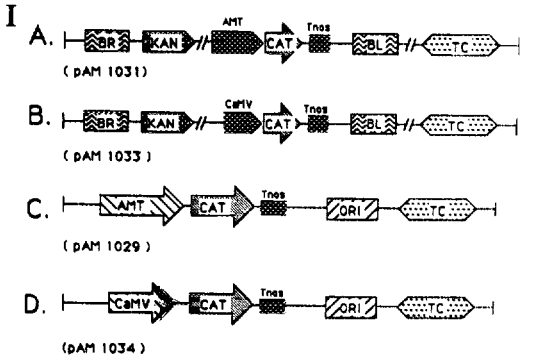


Figure 3. CAT activity in *E. coli* strains and in *Erwinia*, *Pseudomonas* and *Xanthomonas* transformed with plasmid pAM 1032 (A). Ten colonies from each strain were individually tested to obtain average CAT activity (B). See Figure 1 and text for other descriptions.

In order to test the *amt* promoter in plants, *Arabidopsis thaliana* and potato plants were stably transformed by *Agrobacterium* mediated cocultivation method using the binary plasmids pAM1031 and pAM1033. These two plasmids are identical except for the promoters, plasmid pAM1031 contains the *amt* promoter whereas plasmid pAM1033 contains the CaMV 35S promoter (Fig. 4, A&B). The promoters were also compared in two monocot cell lines by determining transient expression in electroporated maize or *Sorghum* protoplasts using plasmids pAM1029 and pAM1034 separately (Fig. 4, C&D). The *amt* promoter generated CAT activity was consistently higher than the *cat* activity obtained by the CaMV 35S promoter. In *Arabidopsis* and potato, the *amt* promoter showed a high level of the *cat* reporter gene expression, levels higher than with the CaMV 35S promoter. The CaMV 35S promoter is considered a strong promoter and is widely used for expression of introduced genes in plants (19). The *amt* promoter was also stronger than the CaMV 35S promoter in maize and *Sorghum* protoplasts.

The promoter is a region recognized by particular consensus sequences, spaced upstream of the coding area of a gene. Interestingly, the *amt* promoter does not contain the conventional TATA and CCAAT eukaryotic sequences or -10 and -35 prokaryotic

I



II

HOST	CONSTRUCT/ PROMOTER	CAT ACTIVITY
Arabidopsis	A (AMT)	137
Arabidopsis	B (CaMV 35S)	53
Potato	A (AMT)	156
Potato	B (CaMV 35S)	29
Maize	C (AMT)	88
Maize	D (CaMV 35S)	19
Sorghum	C (AMT)	92
Sorghum	D (CaMV 35S)	14

Figure 4. CAT activity in transgenic *Arabidopsis* and potato plants and in electroporated maize and *Sorghum* cells. Schematic diagrams of plasmids used are depicted in (I). A total of 15 independently transformed seedlings of each plant was individually tested to obtain average CAT activity (II). For maize and *Sorghum*, 10 million protoplasts were tested for each construct and replicated three times. KAN, kanamycin resistance gene; BR, BL, T-DNA borders. See Figure 1 and text for other descriptions.

sequences although the promoter has demonstrated efficient transcription in both eukaryotic and prokaryotic hosts. In *E. coli*, expression of the *amt-cat* fusion was strong enough to confer resistance to 35 μg/ml chloramphenicol. The level of CAT expression in bacteria was much higher than CAT expression in plants. This is probably due to fact that the *cat* gene was originally isolated from bacteria and its codon usage favors expression in bacteria. Alternatively, since total protein extract was used for CAT assay, it is possible that the ratio of CAT protein is lower in plant extract than in bacterial extract.

An obvious question is, why the *amt* promoter would function so efficiently in higher plants? One possible explanation could be that regulatory proteins of both the *Chlorella*

viruses and higher plants need to deal with high levels of genomic methylation. The methyl groups from these modified bases protrude into the major groove of the DNA (20). Therefore, proteins that regulate gene expression in both higher plants and the *Chlorella* virus must accommodate these methyl groups. Because plant genomes typically have high concentrations of ^{5m}C, e.g. ^{5m}C comprises 30% of the cytosines in maize (20), promoters from viruses with high levels of modified bases may function better in plants.

Most of the bacterial and animal promoters do not function in plants (21). Presently the most commonly used promoters for expressing foreign genes in a variety of higher plants are the CaMV 35S promoter and the T-DNA gene promoters. However, these promoters vary in activity, especially in monocot species (6,21). Therefore, finding additional promoters which function efficiently in dicot and monocot plants is desirable. The *Chlorella* virus *amt* promoter serves as a strong promoter in *Arabidopsis*, potato, maize and *Sorghum*. This same region is also an efficient promoter in a variety of bacteria. The *amt* promoter appears to be unique in that it is strongly expressed both in plants and bacteria indicating that the 5' flanking region is capable of using both eukaryotic RNA polymerase II and bacterial RNA polymerases. The *amt* promoter has potential for use in plant transformation and expression vectors. The *amt* promoter can also be used in binary vectors that can replicate both in *E. coli* and *Agrobacterium*. As the binary vectors are used to transfer part of the plasmid DNA to plant chromosomes for plant transformation, use of the *amt* promoter will allow expression of a given gene both in bacteria and plants.

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REFERENCES

1. Van Etten, J.L., Lane, L.C., and Meints, R.H. (1991) Microbiol. Rev. 55, 586-620.
2. Narva, K.E., Wendell, D.L., Skrdla, M.P., and Van Etten, J.L. (1987) Nucl. Acids Res. 15, 9807-9823.
3. Mitra, A., and An, G. (1989) Mol. Gen. Genet. 215, 294-299.
4. Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. (1987) J. Bacteriol. 169, 5789-5794.
5. An, G., Ebert, P.R., Mitra, A., and Ha, S. B. (1988) In Plant Molecular Biology Manual (S.B. Gelvin, and R.A, Schilperoort, Ed.), Martinus Nijhoff, Dordrecht, The Netherlands, pp. A3,1-12.
6. Benfey, P.N., and Chua, N-H. (1989) Science 244, 174-181.
7. Casadaban, M.J., and Cohen, S.N. (1980) J. Mol.Biol. 138, 179-207.

8. Yanish-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 103-199.
9. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, New York.
10. Raleigh, E.A., Lech, K. and Brent, R. (1989) In Current Protocols in Molecular Biology, (F.M. Ausubel, Ed.), Publishing Associates and Wiley Interscience, New York, Unit 1.4.
11. Bachmann, B.J. (1987) In *E. coli* and *Salmonella typhimurium*, Cellular and Molecular Biology, (F.C. Neidhardt, Ed.), ASM, pp. 1190-1219.
12. Hanahan, D. (1983) J. Mol. Biol. 166, 557-580.
13. Dower, W.J., Miller, J.F., and Ragdale, C.W. (1988) Nucl. Acids Res. 16, 6127-6145.
14. Vehmaanpera, J. (1989) FEMS 61, 165-170.
15. Horsch, R.B., Fry, J.B., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985) Science 227, 1229-1231.
16. Mitra, A., Choi, H.K., and An, G. (1989) Plant Mol. Biol. 12, 169-179.
17. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
18. Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) Mol. Cell Biol. 2, 1044-1051.
19. Sanders, P.R., Winter, J.A., Zarnason, A.R., Rogers, S.G., and Fraley, R.T. (1987) Nucl. Acids Res. 15, 1543-1558.
20. Adams, R.P.L., and Burdon, R.H. (1985) Molecular Biology of DNA methylation. Springer-Verlag, New York. pp. 1-246.
21. An, G. (1986) Plant Physiol. 81, 86-91.